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ANALYSIS OF BIOLOGICAL PRETREATMENT OF RAPESEED STRAW WITH WHITE ROT FUNGI FOR ENZYMATIC HYDROLYSIS

Rasool Ghasemzadeh¹, Mohammad Taghi Hamed Mosavian^{1,}, Afzal Karimi²*

ABSTRACT

The effect of biological pretreatment on the rapeseed straw was evaluated by solid-state cultivation of white rot fungi *Phanerochaete chrysosporium*. *P.chrysosporium* degraded the lignin during the pretreatment, with enzymatic hydrolysis ratios increasing in the pretreated straw (3-fold after a 15 day pretreatment). The samples were identified by XRD, FTIR and SEM. X-ray analysis showed that pretreated samples had higher crystallinity than untreated samples (39,47% for a pretreated sample compared to 33,17% for untreated samples) and FTIR spectroscopy demonstrated that the content of lignocellulose also decreased during the biological pretreatment process. The surface characterization study showed morphological changes in pretreated samples. Moreover, the biological pretreatment slowed a plunge in hydrolysis rate during enzymatic hydrolysis.

Keywords: Cellulose, delignification, hemicellulose, *Phanerochaete chrysosporium*, reducing sugar.

INTRODUCTION

The third widely used oil in the world is rapeseed (*Brassica napus*) oil. In recent years, there has been a significant increase in the application of rapeseed oil to produce biodiesel. According to FAO (2016), rapeseed was cultivated in over 36 million hectares farms around the world in 2014 with Iran's share being about 160000 hectares. After harvesting and separating rapeseed seeds, agricultural residues are usually burned. Due to its lignocellulosic nature, the rapeseed straw can be utilized for fuel ethanol production via a biochemical process. This process includes pretreatment, enzymatic hydrolysis, and fermentation. Enzymatic hydrolysis is an important step in changing lignocellulosic materials into biofuel (Dionisi *et al.* 2015).

The resistant structure of lignin restricts the decomposition of the lignocellulosic materials and their conversion into ethanol (Kumar *et al.* 2006, Lu *et al.* 2009). Pretreatment methods are mainly based on physico-chemical technologies which involve the use of hot water (Zeng *et al.* 2011, López-Linares *et al.* 2014), heat treatment (Boonstra *et al.* 2006), diluted and concentrated acids (Margeot *et al.* 2009, Geddes *et al.* 2010, Li *et al.* 2013) and alkaline (Li *et al.* 2010, Yamashita *et al.* 2010, Tozluoglu 2016).

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Nevertheless, these processes usually require high temperature and operating pressure. Generally, these pretreatments have major drawbacks: (a) the equipment needs to be resistant against corrosion and pressure, (b) most of these processes require the production of environmentally high-risk effluent and (c) they have massive energy consumption (Singh and Chen 2008).

The biological pretreatment by microorganisms and their enzyme systems can degrade lignin and hemicellulose that are already in existence in the lignocellulosic materials at low energy consumption and under mild environmental conditions (Sun and Cheng 2002, Yu *et al.* 2009, Bari *et al.* 2018). White rot fungi are a group of basidiomycetes with unique ability to degrade the structure of lignin and carbohydrates (Yu *et al.* 2010, Salvachúa *et al.* 2011, Nazarpour *et al.* 2013, Camarero *et al.* 2014). These fungi can decompose lignocellulosic materials via the production of two enzymatic systems: (a) hydrolytic system, which produces cellulases and hemicellulases that degrade polysaccharides and (b) oxidative lignolytic system which degrades lignin and opens the phenyl rings. Lignin Peroxidase (LiP), Manganese Peroxidase (MnP) and laccase are the most important of these enzymes (Kumar *et al.* 2006, Yu *et al.* 2009, Castoldi *et al.* 2014, Yao and Nokes 2014, Ansari *et al.* 2016). White rot fungi can produce all or part of these enzymes (Yu *et al.* 2009, Yao and Nokes 2014). Several white rot fungi, such as *Phanerochaete chrysosporium*, *Dichomitus squalens*, *Ceriporiopsis subvermispora*, *Pleurotus ostreatus* and *Coriolus versicolor* have been used for biological pretreatment of biomass. The lignin loss of their cultivation was 10-37% and up to 58% cellulose was degraded; however, only low saccharification ratios (35-40%) were obtained when compared to the saccharification ratios by chemical and physical pretreatment (Itoh *et al.* 2003, Taniguchi *et al.* 2005, Zhang *et al.* 2007). *P.chrysosporium* is one of main candidates used by researchers in several pretreatments (Kumar *et al.* 2006, Sendich *et al.* 2008, Shrestha *et al.* 2008, Lu *et al.* 2009, Singh *et al.* 2011, Zhang *et al.* 2012). The main goal of the present study is the solid-state fermentation of rapeseed straw by *P.chrysosporium* without using any nutrient. The biodegradation patterns of the pretreated straw were also evaluated by chemical component losses, Fourier Transform Infrared (FTIR), Scanning Electron Microscopy (SEM) and X-ray Diffraction Analysis (XRD).

EXPERIMENTAL

Biomass and microorganism

The rapeseed straw was supplied by rapeseed farms in Quchan, Khorasan Razavi Province, Iran in the 2011 crop year. To remove soil particles and unwanted materials, the rapeseed straw was washed with water and dried in laboratory environment. Then, it was oven dried at 60°C for 24 h to reach a constant weight. The dried rapeseed straw was grounded, passed through an 18-mesh sieve, packed in nylon containers for further use and stored at room temperature.

The lyophilized *P.chrysosporium* (ATCC 24725) was purchased from Iranian Regional Center for Collection of Industrial Fungi and Bacteria. The organism was propagated on potato dextrose agar (PDA) plates at 30°C for one week and the spores were preserved in dark at 4°C. Spore suspensions were prepared by washing the agar surface by distilled water and were then diluted so that an absorbance of 0,5 at 650 nm was obtained in a 1 cm path length cuvette, as suggested by Sedighi *et al.* 2009. There were 2×10^6 spores per ml of the suspension solution. To ensure the appropriate function of the fungus, a new culture medium was prepared every two months.

Biological pretreatment by *P.chrysosporium*

In the first step, 5 g of the rapeseed straw was placed into a capped 250 ml jar and sterilized in the autoclave at 121°C for 15 min and then left to cool down at room temperature. A 2,5 ml of the spore suspension was added to the jar, and moisture of the medium was increased to $80\% \frac{w \text{ water}}{w \text{ total}}$ using sterile distilled water. The cultures were capped and incubated at 37°C for 5, 10 and 15 days. All cultures were grown in triplicate. In all cases, the rapeseed straw treated in the same conditions without fungal inoculation was used as the control.

Enzymatic hydrolysis

Enzymatic hydrolysis was performed in 250 ml of Erlenmeyer flask with 1 g of the pretreated sample and 100 ml of 0,05M sodium citrate buffer. A certain amount of cellulase (10 FPU/g, from Sigma) was added to each flask and the reaction mixtures were incubated in the shaker incubator at 50°C and 150 rpm for a maximum of 56 h. Each time point was analyzed for reducing sugar in the triplicate and an average value was reported.

Determination of reducing sugar

The total reducing sugar was determined by 3,5-dinitrosalicylic acid (DNS) method using glucose as the standard (Miller 1959). A 0,1 ml of the enzymatic hydrolysis solution and 0,9 ml of distilled water were poured into the test tube and then 3 ml of DNS solution was added to the tube. The mixture was placed in a hot water bath for 5 min and cooled down to room temperature. Then, 7 ml of distilled water was added to the tube, and the absorption occurred at wavelength of 540 nm. The absorbance readings were then converted into equivalent sugar concentration (mg/ml) using a standard glucose curve. The hydrolysis ratio was calculated using Equation 1:

$$\text{Hydrolysis ratio}(\%) = \frac{\text{Amount of reducing sugar produced} \times 0,9}{\text{Amount of cellulose} + \text{amount of hemicellulose}} \times 100 \quad (1)$$

Chemical analysis of rapeseed straw

Rapeseed straw was dried at 60 °C to reach a constant weight. Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), and Acid Detergent Lignin (ADL) were determined based on the procedure proposed by Van Soest 1963. The amount of hemicelluloses, cellulose and Klason lignin were calculated by measuring the difference between NDF and ADF content, ADF and ADL content and ADL and ash content, respectively. The detail process was as follows:

About 1 g of dried rapeseed straw and 100 ml of neutral detergent solution with Sodium lauryl Sulfate (SDS) and disodium Ethylene Diamine Tetra Acetate (EDTA) were added into a flask to determine NDF. The mixture was then heated to reach the boiling point within 5-10 min and was boiled for one hour. Then, the hot mixture was filtered; the residues were washed with a minimum of 500 ml of hot distilled water and 10 ml of acetone and dried at 105 °C to reach a constant weight. The amount of NDF was determined by Equation 2:

$$\text{NDF}(\%) = \frac{\text{Weight of dried residue after treatment with neutral detergent solution}}{\text{Sample weight}} \times 100 \quad (2)$$

Acid detergent solution and Cetyl Tri-methyl Ammonium Bromide (CTAB) and 0,5 M H₂SO₄ were used to determine ADF (Equation 3) in a similar way to NDF determination.

$$\text{ADF}(\%) = \frac{\text{Weight of dried residue after treatment with acid detergent solution}}{\text{Sample weight}} \times 100 \quad (3)$$

For the determination of ADL, the solid material obtained from ADF was digested with 72% H₂SO₄ for 3 h and then filtered. The residue was completely washed by hot distilled water until it was acid free in pH paper. The residue was dried at 105 °C to reach a constant weight (Equation 4).

$$ADL(\%) = \frac{\text{Weight of dried residue after treatment with 72\% sulphuric acid}}{\text{Sample weight}} \times 100 \quad (4)$$

The ash was determined as the residue after treatment in the muffle at 600 °C for 24 h (Equation 5).

$$\text{Ash}(\%) = \frac{\text{Ash weight}}{\text{Sample weight}} \times 100 \quad (5)$$

X-ray diffraction analysis

The Crystallinity Index (Crl) of untreated and treated rapeseed straw by *P.chrysosporium* was collected by an X-ray diffractometer in a BRUKER (Germany). All samples were scanned in the range of $2\theta = 0-40^\circ$ at a speed of $1^\circ/\text{min}$ at 40 kV and 40 mA. The radiation used was Cu ($1,54\text{\AA}$) and CrI was calculated according to Equation 6:

$$\text{Crystallinity Index (Crl)}\% = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \quad (6)$$

Where I_{002} is the maximum intensity of peaks at 2θ between 22 and 23° , and I_{am} is the minimum intensity of peaks in 2θ between 18 and 19° (Segal *et al.* 1959).

Fourier Transform Infrared spectroscopy (FTIR) analysis

The FTIR spectra were obtained from direct transmittance using the KBr pellet technique through a Perkin Elmer 100 FTIR spectrometer. All spectra ($4000 - 400 \text{ cm}^{-1}$) were measured at a resolution of 4 cm^{-1} and 64 scans per sample.

Scanning Electron Microscopy (SEM) analysis

Scanning Electron Microscopy (SEM) was used to study the structural changes of rapeseed straw after biological pretreatment. The samples were dried and coated with gold. The SEM images were taken with a LEO 1450 VP microscope at 15 kV.

Data treatment

Equation 7, an empirical model (Ohmine *et al.* 1983) was used for data treatment of enzymatic hydrolysis.

$$X = \frac{1}{k} \ln(1 + V_0 kt) \quad (7)$$

Where X= hydrolysis ratio (%), V_0 = initial hydrolysis rate (%/h), t = hydrolysis time and k = the rate constant for the retardation process.

According to Equation 7, V_0 and k were determined by regression analysis of the experimental results. Data fit was performed by MATLABR2013a software.

Selectivity value

Selectivity value is the ratio of lignin degradation to cellulose reduction and it was calculated according to Equation 8:

$$Selectivity\ value = \frac{Lignin\ loss}{Cellulose\ loss} \tag{8}$$

RESULTS AND DISCUSSION

The effect of biological pretreatment on rapeseed straw chemical composition

Lignin is an important compound found in plant cell walls that limits chemical and biological decomposition and hydrolysis. The reduction of lignin content leads to the exposure of the crystalline structure of cellulose and facilitates substrate access by hydrolytic enzymes (Sun and Cheng 2002). The effect of biological pretreatment on the rapeseed straw after cultivation with *P.chrysosporium* is shown in Table 1. All components declined over time. In this study, compounds of the rapeseed straw included lignin (23,9%), cellulose (35,08%), and hemicelluloses (33,83%). The largest amount of lignin degradation was observed after 15 days (6,18%). This level of lignin removal can be attributed to the maximum production of lignolytic enzymes at the 15th day (Ghasemzadeh *et al.* 2015). The results are in line with previous studies in which biological pretreatment of lignocellulosic materials had been investigated by white rot fungi (Xu *et al.* 2010, Singh *et al.* 2011, Deswal *et al.* 2014).

Table 1. Losses of different components of the rapeseed straw pretreated by *P. chrysosporium**.

Pretreatment time (day)	Selectivity	Component loss (%)		
		Lignin	Cellulose	Hemicellulose
5	1,17	1,08±0,32	0,92±0,72	2,63±0,22
10	1,48	4,36±0,71	2,94±0,38	6,44±0,34
15	1,83	6,18±0,56	3,37±0,41	7,27±0,53

*Results show the mean value of three replicates ± standard deviation.

Given that the purpose of this study was pretreatment of rapeseed straw without any nutrient, the resulted values of lignin, cellulose and hemicellulose degradation and also the enzymatic hydrolysis of pretreated straw were less than similar studies which have mostly used more time, nutrients and so on. Certainly, the type of biomass, pre-treatment time and type of the microorganism are very effective factors. Different studies by other researchers are shown in Table 2.

The ratio of lignin degradation to cellulose reduction (selectivity value) can be considered as a function of delignification efficiency. That is, high selectivity implies good prospects for preferential delignification whereas low selectivity indicates massive removal of cellulose during pretreatment (Nazarpour *et al.* 2013). Selectivity value was used to evaluate the selective lignin-degrading ability. As shown in Table 1, selectivity values rose with an increase in the pretreatment time and it reached its maximum on the 15th day (1,83). Nevertheless, some reports suggest that selectivity values drop

with increased pretreatment time. Also, non-selective degradation occurred with an increase in the pretreatment time (Ferraz *et al.* 2000, Yu *et al.* 2009, Nazarpour *et al.* 2013). This subject was not investigated in the present study.

Table 2. Component losses of some feedstocks pretreated by white rot fungi.

Microorganism	Lignin loss (%)	Hemicellulose loss (%)	Cellulose loss (%)	Biomass	Ref.
<i>C. subvermispora</i> (90 day)	45,06	42,08	9,50	Rubberwood	(Nazarpour <i>et al.</i> 2013)
<i>T. versicolor</i> (90 day)	34,40	37,90	32,06		
<i>P. chrysosporium</i> (14 day)	35,053	60,05	43,26	Cotton stalk	(Shi <i>et al.</i> 2009)
<i>Irpex lacteus</i> (150 day)	68,42	76,92	76,71	Corn stover	(Xu <i>et al.</i> 2010)
<i>Pleurotus florida</i> (60 day)	36	45,90	18,30	Corn straw	(Zhong <i>et al.</i> 2011)
<i>Trametes hirsute</i> yj9 (14 day)	41,74	52,93	11,97	Corn stover	(Sun <i>et al.</i> 2011)
(28 day)	59,50	71,40	27,26		
(42 day)	71,99	77,84	34,06		

Characterization of pretreated rapeseed straw.

Characterization of pretreated rapeseed straw

The FTIR spectroscopy of the pretreated rapeseed straw (15th day) and untreated straw (0th day) is shown in Figure 1. A strong hydrogen bonded (O-H) stretching absorption is observed at around 3420 cm^{-1} , while bonds at 2920 and 2850 cm^{-1} represent C-H anti-symmetric and symmetric stretching, respectively. Moreover, the existing peaks of $1800\text{--}700\text{ cm}^{-1}$ indicate the following bonds: 1640 cm^{-1} for absorbed O-H and conjugated C-O; 1440 cm^{-1} for destroyed C-H in lignin; 1380 cm^{-1} for C-H deformation in cellulose and hemicellulose; 898 cm^{-1} for C-H deformation in cellulose and peaks between 1200 and 1000 cm^{-1} represent C-O stretching and deformation in cellulose, lignin, and residual hemicellulose bonds (Zhang *et al.* 2012, Nazarpour *et al.* 2013). There are many similarities between FTIR spectra of the pretreated rapeseed straw by *P.chrysosporium* (15th day) and the untreated rapeseed straw (0th day), which indicate the selective removal of carbohydrate components. The intensity of absorption has considerably reduced with an increase in the pretreatment time, indicating the solubilization of constituents of lignocelluloses fraction. The elimination or reduction of absorption intensity at peaks of 1060 , 1160 and 898 cm^{-1} with increased pretreatment time indicate the possibility of destroying lignocellulosic structure with an increase in time. According to FTIR spectra, the permitted incubation period for fungal treatment should be increased to facilitate solubility of lignocellulose and further breakdown.

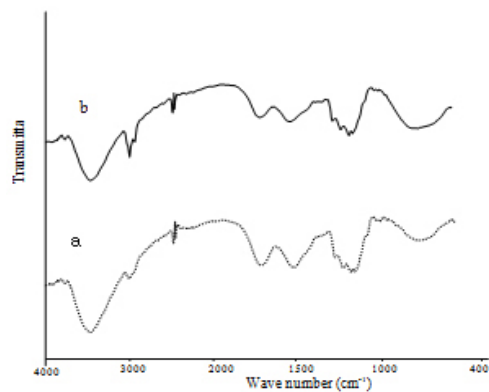


Figure 1. FTIR spectra of rapeseed straw before and after pretreatment by *P.chrysosporium*: (a) Before pretreatment (...), (b) 15 days after pretreatment (-).

The main compounds of lignocellulosic biomasses are cellulose, hemicellulose and lignin, with cellulose being surrounded by lignin and hemicelluloses (Sun and Cheng 2002, Chandra *et al.* 2007). Hemicellulose and lignin are amorphous components, while cellulose is considered to be a crystalline component (Nazarpour *et al.* 2013). Given the impossibility of complete separation of cellulose from other components of fibers, the direct measurement of cellulose crystallinity in biomass is not possible (Zhao *et al.* 2008). Currently, the measurement of crystallinity index by X-ray is the best way of estimating the effectiveness of pretreatment in the crystallinity of biomass (Kumar *et al.* 2009). The intensities of amorphous and crystalline regions were measured at 2 θ (18°) and 2 θ (22 and 23°), respectively. Table 3 shows the crystallinity index for untreated and pretreated rapeseed straw by *P.chrysosporium*. As can be seen, the crystallinity index increased during pretreatment time so that after 15 days it reached 39,47%, whereas this figure was 33,17% in the untreated rapeseed straw. The increased crystallinity index might be attributed to the degradation and modification of hemicellulose and lignin, as reported in previous studies (Kim and Lee 2005, Jeoh *et al.* 2007, Nazarpour *et al.* 2013). Therefore, the augmented crystallinity index in pretreated samples suggests greater exposure of cellulose after biological pretreatment. This has been discussed in a number of previous reports (Zhang *et al.* 2012, Nazarpour *et al.* 2013).

Table 3. Crystallinity Index of untreated and fungal-treated rapeseed straw by *P.chrysosporium*.

Samples	CrI (%)		
	5 days	10 days	15 days
Untreated	33,17	33,17	33,17
Fungal treated	34,23	37,15	39,47

The morphologies of samples before and after pretreatment were examined by SEM. As shown in Figure 2, the untreated rapeseed straw had a compact structure with a smooth flat surface area and a partial porosity, while the fungal-pretreated rapeseed straw had a partially broken or degraded face and many small pores and sponge-like structures were observed after pretreatment by *P.chrysosporium*. By hand-touching, it was found that the pretreated rapeseed straw was significantly softer and looser than the untreated ones. This conversion could be due to fungal growth or effects of extracellular enzymes secreted by the fungus. The structural changes of fungal-pretreated rapeseed straw can provide larger accessible surface area for the enzymatic hydrolysis and result in enhanced digestibility of the pretreated

substrate.

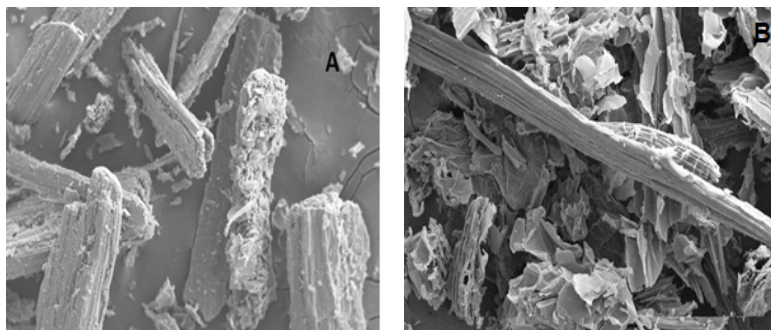


Figure 2. SEM images of the rapeseed straw before and after pretreatment: A) untreated and B) pretreated by *P.chrysosporium* after 15 days.

Enzymatic hydrolysis of rapeseed straw

To evaluate the effect of pretreatment by *P.chrysosporium* on the enzymatic hydrolysis of pretreated rapeseed straw, hydrolysis ratio was determined after enzymatic hydrolysis for 0-56 h (Figure 3). As can be seen, the untreated rapeseed straw was more resistant to enzymatic hydrolysis, yielding a hydrolysis ratio of 2,2% after 56 h of hydrolysis. The hydrolysis ratio increased with the pretreatment time. The highest hydrolysis ratio of the pretreated rapeseed straws after 5, 10, and 15 days were 3,6; 4,8; and 6,6%, respectively. The results indicate that the enzymatic hydrolysis is significantly affected by the pretreatment time. As the pretreatment time increases, delignification and hemicellulose removal is intensified, leading to greater availability of enzymes to cellulose and consequently improved digestibility of the cellulose (Öhgren *et al.* 2007, Nazarpour *et al.* 2013).

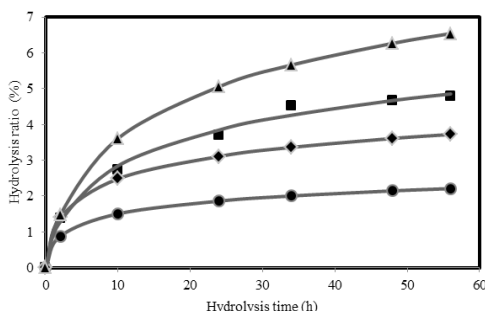


Figure 3. Time course of hydrolysis ratio (%) during the hydrolysis of rapeseed straw (a) untreated (●) and (b) pretreated by *P.chrysosporium* after 5 (◆), 10 (■) and 15 (▲) days.

Equation 7 was used to characterize hydrolysis kinetics by regression analysis of the experimental data. As shown in Figure 3 (dotted curve), a good agreement with the experimental results was observed in the regression curves. According to Table 4, the biological pretreatment of rapeseed straw by *P.chrysosporium* increased the initial hydrolysis rate V_0 on the 5th day. After 5 days of pretreatment, there was a 1,38-fold increase in the initial hydrolysis rate V_0 , but it began to decline with increased pretreatment time. However, the rate constant for the retardation process (k) dropped after the pretreatment, such that after 15 days, k value decreased by 4,4 fold, indicating the effect of biological pretreatment on slowing down the decline in hydrolysis rate during enzymatic hydrolysis. Similar results have been reported by Yu *et al.* 2009.

Biological pretreatment leads to the degradation of lignin and consequently increased pore size in the substrate, which provides greater accessible surface area to cellulose. As depicted in Table 1, the degree of lignin removal change depends on the pretreatment time. As illustrated in Figure 4, there is a linear relationship between the lignin degradation and hydrolysis ratio ($R^2 = 0,951$). As lignin degradation increased from 0 (untreated) to 6,18% the hydrolysis ratio decreased from 2,2 to 6,62%. Some reports have demonstrated the correlation between hydrolysis ratio and lignin degradation of

biological pretreated lignocellulosic biomass (Wan and Li 2010, Nazarpour *et al.* 2013), which indicate the possibility of cellulose digestibility by preferential degradation of lignin.

Table 4. Kinetics parameters of enzymatic hydrolysis of untreated and fungal-treated rapeseed straw by *P.chrysosporium* based on Equation 7.

Pretreatment time (day)	V ₀ (%/h)	K	R ²	SSE
0	1,530	2,415	0,991	0,036
5	2,114	1,368	0,996	0,040
10	1,133	0,817	0,994	0,114
15	1,136	0,547	0,997	0,095

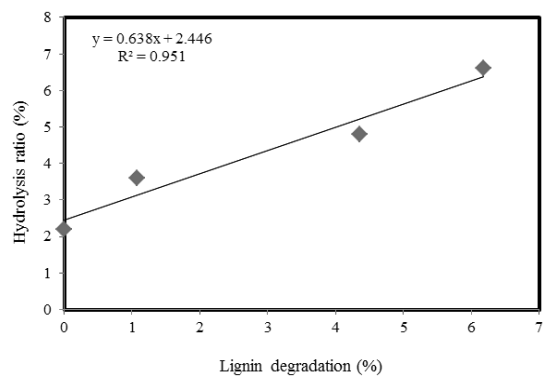


Figure 4. Effect of lignin degradation on the enzymatic hydrolysis of rapeseed straw pretreated by *P.chrysosporium*. Hydrolysis ratio (%) was obtained after 56 h of enzymatic hydrolysis.

CONCLUSIONS

The results suggest that the enzymatic hydrolysis of the rapeseed straw can be enhanced by biological pretreatment with *P.chrysosporium*. The X-ray analysis exhibited higher crystallinity of all pretreated samples compared to samples in the control group. Nevertheless, the highest crystallinity of biological pretreated sample was observed after 15 days. It increased from 33,17% for the untreated rapeseed straw to 39,47% for the pretreated sample after 15 days. The lignin degradation by *P.chrysosporium* was confirmed using FTIR spectroscopy. The results of FTIR indicated the preferential nature of this white rot fungi. Scanning Electron Microscopy (SEM) showed structural changes in the pretreated rapeseed straw, such that the surface of pretreated rapeseed straw was partially broken and many pores were developed. In addition, the pretreatment slowed down hydrolysis rate during enzymatic hydrolysis, such that after 15 days, a 4,4-fold decrease in k value was observed.

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